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TEMPERATURE-ACCLIMATIZATION OF LISTERIA MONOCYTOGENES ACCORDING TO THE CHANGES IN THEIR PLASMATIC MEMBRANE STABILITY

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ABSTRACT
PURPOSE: To study the effect of growth temperature, T_{gr}, on the thermal resistance of *Listeria monocytogenes*, strains № 1, 4, 5 and *Listeria innocua*, strain № 6, and on thermal stability, T_g, of their plasmatic membranes. METHODS: 1) measurement of the survival fraction of bacteria after exposure to overcritical temperature (54°C), and 2) measurement of the T_g temperature representing the ability of cells to retain the cytosolic ions at hyperthermia. RESULTS. The T_g had low and constant values (about 57°C) in bacteria grown at the 10 – 37°C interval of moderate temperatures but strongly increased (67 – 70°C) at both temperature extremes of growth (6°C and 41°C). This result was supported by the data for thermal resistance, which was greater in bacteria grown at low T_{gr} (9°C) compared to that of cells cultivated at moderate T_{gr} (30°C). CONCLUSION: the thermal resistance and plasma membrane stability of *L. monocytogenes*, as represented by the T_g temperature, changed significantly depending on weather the bacteria was cultivated at the moderate or extreme temperatures of growth.

Key words: Thermal resistance, permeability barrier stability, temperature acclimation, *Listeria monocytogenes*.

INTRODUCTION

The genus Listeria includes 6 different species (*L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*), of which *L. monocytogenes* is consistently associated with illness in human (1) and farm animals (2). The greatest threat of listeriosis for humans is from ready-to-eat products since the bacteria have relatively high heat resistance and ability to multiply in refrigeration temperatures (3).

The thermal resistance of bacteria must be considered when quantifying and modelling microbial inactivation during thermal processing of foods. It is frequently expressed in terms of the D value (decimal reduction time in minutes required for the heat destruction of one log of bacteria population through exposure to specified temperature) and in the *Z* value (the change in temperature necessary to cause a tenfold change in the *D* value) (4). It is broadly accepted that low-temperature long-time (51.6°C, 97 min) or high-temperature short-time (62.7°C, 1.06 min) pasteurization of *L. monocytogenes* both equally reduce its viability (5, 6).

Some strains exhibit strong temperature acclimatization, i.e., different thermal resistance depending on the temperature of growth of culture. This should be taken under consideration in choosing the model for their heat inactivation. In addition, prior heat shock (48°C) has induced increased heat resistance in *L. monocytogenes*, a thermotolerance, to subsequent higher heat treatments to 55°C (7). The extent to which cells become thermotolerant after a heat shock depends on a number of factors including the growth temperature (4 and 37°C) prior to heat shock (8). This adaptive response of pathogenic bacteria to heat is believed responsible for the

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frequent occurrence of deviations (tails and shoulders) during heat treatments that are observed in the exponential model of microbial inactivation (9). These deviations from log-linear kinetic, especially encountered under mild heat treatments, mean that prediction of food safety can no longer rely upon *D* and Z values (9). Therefore a more mechanistic approach is needed for more accurate predictions of thermal inactivation. Prerequisite to this model are thorough studies to understand how *L. monocytogenes* and other pathogens adapt their cellular physiology to overcome heat and other stresses.

Based on the above considerations our report is aimed at studying the temperature acclimatization of *L. monocytogenes* as expressed by the changes in their thermal resistance over the whole interval of growth temperatures. The permeability barrier function of cells is a vital requisite and the ability to maintain it emphasises resistance to deleterious conditions. Generally, the membranes of cells undergo an ethanol facilitated permeabilization at high temperatures (10), which in some cases has been related to the thermal resistance of cells (11, 12). The thermal resistance was determined by the classical colony forming test of heat-challenged bacteria and assessed by the thermal stability test of plasmatic membranes.

MATERIALS AND METHODS

1. Test microorganisms. L. monocytogenes was obtained from the National Reference Center of Food Safety at the National Diagnostic and Research Veterinary Institute, Sofia, Bulgaria. Prior to growth, stock cultures of bacteria were maintained in liquid nutrient medium with glycerol, placed in criovials under liquid nitrogen (- 196°C). The following strains were used:

N1 – Listeria mononocytogenes H34B

N4 – Listeria monocytogenes 60 (an isolate of sausage)

N5 – L. monocytogenes (MB IMCC 8632) - referent strain

N6 – Listeria innocua 27

2. Preparation of bacteria cells. The stock suspension of indicated bacterial strain was prepared inoculating bacteria in Caso broth TSAYE at the optimal temperature of 30°C. The maximal cell concentration was obtained after 18-20 hours growth as determined spectrophotometrically at 600 nm.

3. Temperature acclimatization of bacteria. 50 μl of the obtained stock bacterial suspension were inoculated into 5 ml of the same Caso broth TSAYE (Merk). The bacteria were allowed to grow at the indicated growth temperature T_{gr}, controlled by a thermostat (Dry thermostatic cell chamber TLSO 80, ETNA producer, Bulgaria). To determine the cellular resistance to heat, only cells harvested from cultures in the early stationary phase were used. The stationary phase was attained after 14 days growth at 2,5°C, 7 days growth at 6°C, 18-20 hours growth at 30°C, and so on. Following incubation the bacteria were tested for their resistance against thermal challenge and for changes in the thermal stability of their cytoplasmic membranes. The overall structure of the cell envelope of *L. monocytogenes* is shown in **Fig.1** (13).

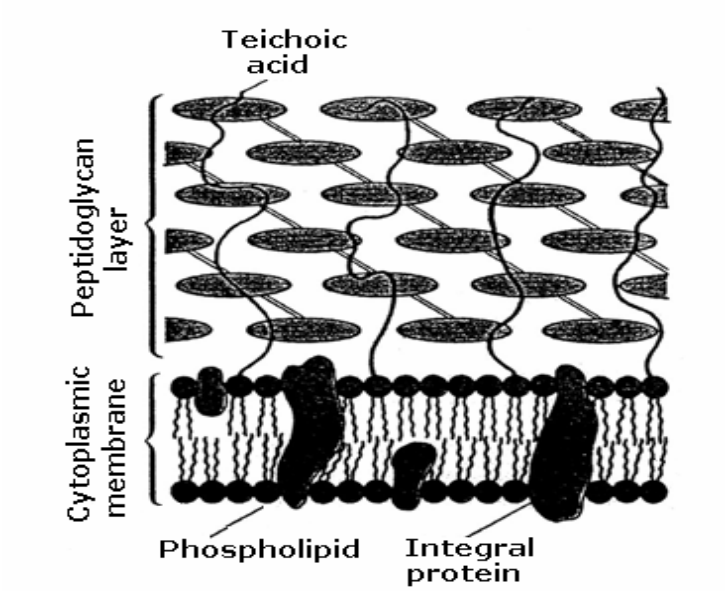


Figure 1. Cell envelope of Gram positive L. monocytogenes.

4. **Hyperthermic killing of cells and determination of survival fraction**. Thermal resistance of acclimated bacteria was determined against a stress temperature (54°C), which was significantly higher than the maximal temperature of growth (43°C) for these bacterial strains. 10 ml of cell culture was placed in water bath at 54°C. During the heating (the warm up time was excluded), aliquots of 1 ml were taken at the indicated time intervals and duplicate probes of 0.1 ml were plated on agar, supplemented with Caso broth TSAYE, to determine the fraction of cells (in CFU/ml) remaining intact. After 48 h growth at 30°C, the colonies formed were counted and the survival fraction determined (14). Survivor curves were obtained by plotting mean counts (log N) from duplicate plate samples against treatment time at 54°C.

5. **Test of the thermal stability of plasma membranes** (12). Prior to usage the cells were isolated by centrifugation (7000xg, 5 min) from the growth medium and once washed in 0.5 ml low-salt medium that contained 3 mM NaCl and 70 mM sucrose. The washed cells were isolated and resuspended in 0.1 ml of the same medium to impose strong transmembrane gradient of ion concentration. The final concentration of cells was between 5 and 30 g (wet weight) cells per litre medium. The final bacterial suspension was heated with constant heating rate (2°C/min) from 20°C to about 70°C and data for temperature and electric conductivity (20 kHz) of the suspension were collected and transmitted to a computer. These data were further processed to obtain the temperature derivative of conductivity, which was presented as a function of the transient temperature (**Fig. 1** for example). This was the so called derivative thermogram of suspension conductivity.

During the heating the ion permeability of the tested suspension became strongly activated within a narrow temperature interval about a specific high temperature, T_g, causing the collapse of the transmembrane ion-concentration gradient. This thermally induced dissipation of ion gradient was detected as a peak on the conductivity thermogram, allowing a precise determination of T_g. The T_g was determined by the top peak temperature where the outward ion flow had the maximal value. The T_g corresponded to the ability of bacteria to preserve its cytosolic content of conductive material at high temperatures, i.e., to the thermal stability of plasmatic membranes. During repeated experiments with samples of the same probe of cells, the reliability of determining T_g was about +/- 0.3°C.

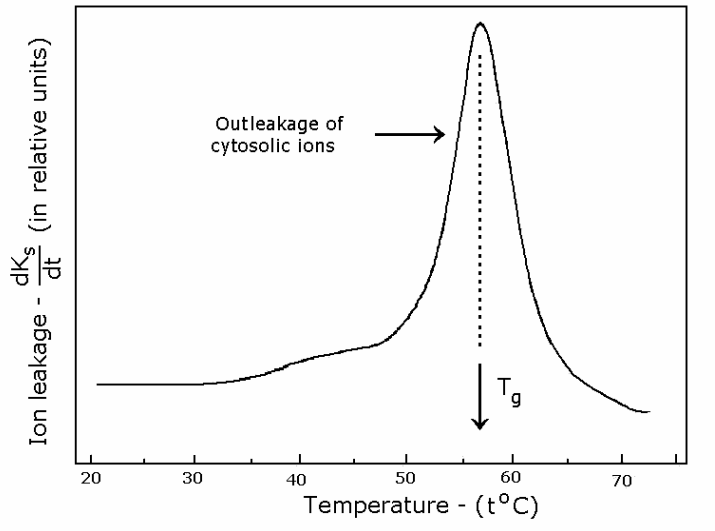


Figure 2. Temperature profile of the conductivity derivative dK_s/dt of a suspension of L. monocytogenes, strain N4. The top temperature T_g of the right peak was considered representative of the thermal stability of membranes (ability to retain the cytosolic ions at hyperthermia). The suspension media contained 70 mM sucrose and 3 mM NaCl. The suspension contained 10 mg/ml (wet weight) of bacteria and was heated with 2°C/min.

RESULTS

Fig. 2 shows the derivative conductivity thermogram of once washed *L. monocytogenes*, strain N4, suspended in a low electrolyte medium. This is a typical record of the first derivative of the electrical conductivity exhibiting a sharp peak at the T_g temperature. Specific sensitivity of the applied method allowed us to conclude that this peak corresponds to out leakage of cytosolic conductive material. Similar thermograms were obtained with all other bacterial strains used. During the second heating of the same suspension the peak, centred at the T_g temperature, did not show up on the repeated thermogram. This demonstrated complete irreversibility of the high temperature process in which a denaturation of membrane protein was apparently involved. Based on the above the top temperature T_g was further considered as quantitative measure of the membrane ability to retain the transmembrane gradient of ion concentration.

Previous studies (12) have demonstrated that the amplitude of this peak depended on the magnitude and direction of the ion-concentration gradient that was imposed across the membranes of whole cells and the membrane vesicles isolated from them. Thus, this peak represented thermally-induced alteration of cellular membranes accompanied by activation of ion permeability and consequent breakdown of permeability barrier. This barrier breakdown event took place at the same temperature interval where a protein denaturation had been microcalorimetrically detected in isolated membranes of *Acholeplasma laidlawii* (15) and *Escherichia coli* (16). This coincidence possibly associated the breakdown of permeability barrier with the concomitant structural changes in membrane proteins. Hence, this peak was apparently related to the thermal stability of permeability barrier and membrane proteins involved.

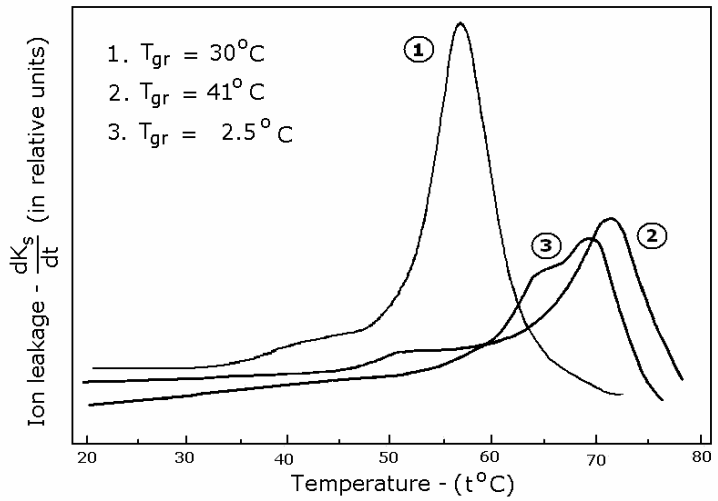


Figure 3. Derivative thermogram of L. monocytogenes, strain N4, as affected by the growth temperature of bacteria. The cells were grown at the indicated temperature, isolated and studied as described for Figure 2.

Previous study of a *Str. faecalis* strain has demonstrated that the T_g temperature and thermal resistance of bacteria both strongly changed in concert depending on the changes in growth conditions (17), including changes in growth temperature (12). Similar result has been reported for the thermal resistance of *Aeromonas hydrophila* (18). Fig. 3 shows that the T_g temperature of *L. monocytogenes*, strain №4, depended on the growth temperature; it had higher values in cells grown at the temperature extremes (2.5°C and 41°C) compared to that in cells grown at the optimal temperature of growth (30°C). Similar result was obtained for the thermal resistance of the same strain, cultivated at different growth temperatures. Fig. 4 demonstrates that thermal resistance of *L. monocytogenes*, strain №4,

changed in concert with the thermal stability of their plasmatic membranes; in cells cultivated at the temperature extremes, it had higher values in comparison to the same parameter of cells grown at the optimal temperature. This finding compelled us to conclude that the T_g temperature of *L. monocytogenes*, strain №4, was a parameter closely related to its thermal resistance and both represented the ability of bacteria to withstand the challenges of high temperatures such as those during pasteurization of pathogen bacteria.

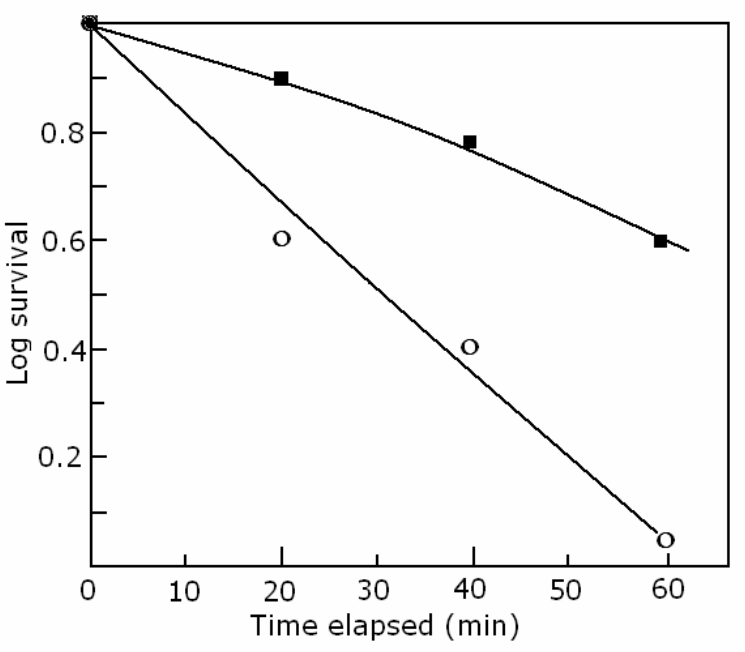
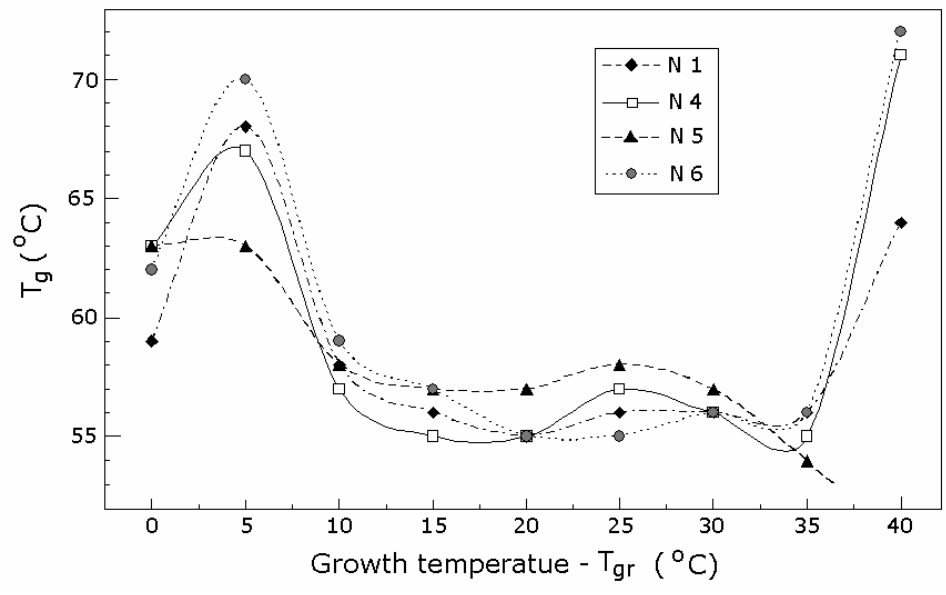


Figure 4. Survivor curves of L. monocytogenes, strain N 4. The cells were acclimated at 30 ^{o}C (○) and 6 ^{o}C (■), exposed to 54 ^{o}C for the indicated time intervals and the survival fractions counted.

Based on the above conclusion we conducted thorough investigation of the effect of growth temperature on the T_g temperature, membrane stability, for the three strains of *L. monocytogenes* and the *L. innocua* strain. The result is displayed in Fig. 5. The four curves represent the acclimatization of bacteria to different temperatures over the entire 2^{o}C - 42^{o}C temperature interval of growth as expressed by the changes in the thermal stability of plasma membranes. There was a broad intermediate interval (10^{o}C – 35^{o}C) of growth temperatures where the T_g temperature had low and almost constant value for the four strains. The mean value of T_g in this region of non-stress temperatures was about 57-58^{o}C which is about 16^{o}C greater than the maximal temperature of growth (41^{o}C). The latter finding is in line with recent report that the T_g temperature for many cell types is about 17^{o}C above their maximal temperature of growth (19).

Surprisingly, the T_g temperature strongly increased at both temperature extremes of growth, 41^{o}C and about 5^{o}C. Only the strain № 5 had not the ability to increase its T_g at 41^{o}C and, consequently, its growth at this temperature was poor. The rise of T_g was very strong, about 10^{o}C that corresponds to a huge increase in the thermal stability of plasma membranes and thermal resistance of bacteria. This finding indicates that the strains of *L. monocytogenes* and of *L. innocua* under study have strongly expressed ability to change its physiology during growth at the temperature extremes resulting in an intense increase in the stability of permeability barrier function and thermal resistance of bacteria cells.

This intense adaptation capacity of *L. monocytogenes* could be related to its ability to grow at both refrigeration and high temperatures. Hence, the applied method for detecting the T_g temperature, in combination with other methods, could be very useful in investigating the molecular mechanism allowing these pathogens to adapt and grow at very unfavorable temperatures.



5.

Figure 5. Effect of the growth temperature on the thermal stability of the plasmatic membranes of L. monocytogenes (№ 1, 4 and 5) and of L. innocua (№ 6). After cultivation at the indicated growth temperature, T_{gr}, the cells were isolated and the thermal stability of their membranes, T_g, determined as described for Figure 2.

CONCLUSION

The paper reports strong changes in *L. monocytogenes* thermal resistance and stability of plasmatic membrane during cultivation at different growth temperature that could be used in future studying the adaptation potential of this pathogen.

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